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RESEARCH COMMUNICATION

Tyrosine phosphorylation is involved in receptor coupling to phospholipase D but not phospholipase C in the human neutrophil

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The tyrosine kinase inhibitors ST271, ST638 and erbstatin inhibited phospholipase D (PLD) activity in human neutrophils stimulated by fMet-Leu-Phe, platelet-activating factor and leukotriene B_4. These compounds did not inhibit phorbol ester-stimulated PLD, indicating that they do not inhibit PLD *per se*, but probably act at a site between the receptor and the phospholipase. In contrast, the protein kinase C inhibitor Ro-31-8220 inhibited phorbol 12,13-dibutyrate- but not fMet-Leu-Phe-stimulated PLD activity, arguing against the involvement of protein kinase C in the receptor-mediated activation of PLD. ST271 did not inhibit Ins(1,4,5)P_3 generation, but did inhibit protein tyrosine phosphorylation stimulated by fMet-Leu-Phe. The phosphotyrosine phosphatase inhibitor pervanadate increased tyrosine phosphorylation and stimulated PLD. These results suggest that tyrosine kinase activity is involved in receptor coupling to PLD but not to PtdIns(4,5)P_2-specific phospholipase C in the human neutrophil.

INTRODUCTION

The hydrolysis of phosphatidylcholine by phospholipase D (PLD) is now recognized as an important source of second messenger molecules in many cell types [1]. The mechanism by which receptors couple to PLD is poorly understood, but existing evidence suggests that a variety of different mechanisms may occur [2]. Phorbol esters stimulate PLD in many tissues through the activation of protein kinase C (PKC) [3–5], and increases in intracellular Ca^{2+} resulting from treatment with the calcium ionophore A23187 also activate PLD [6,7]. The possible involvement of both Ca^{2+} and PKC in the activation of PLD by receptor agonists has been rationalized by the theory that hydrolysis of PtdIns(4,5)P_2 by PtdIns(4,5)P_2-specific phospholipase C (PtdInsP_2-PLC) is a necessary prerequisite for PLD activation [8]. The products of PtdInsP_2 hydrolysis, namely Ins(1,4,5)P_3 and diacylglycerol, are known to initiate Ca^{2+} mobilization and activate PKC respectively. Consistent with this model of receptor-mediated PLD activation is the observation that agonists which stimulate PLD in the neutrophil also stimulate PtdInsP_2-PLC. Indeed, activation of PtdInsP_2-PLC precedes activation of PLD in fMet-Leu-Phe-stimulated neutrophils [9].

The coupling of receptors to PtdInsP_2-PLC is mediated both by GTP-binding proteins and through protein tyrosine phosphorylation [10,11]. In the neutrophil, the activation of PtdInsP_2-PLC and PLD by fMet-Leu-Phe is sensitive to pertussis toxin [7,12], thus implicating GTP-binding proteins in the coupling of the fMet-Leu-Phe receptor to both phospholipases. In electropermeabilized neutrophils, activation of GTP-binding proteins by guanosine 5′-[γ-thio]triphosphate has been shown to stimulate both superoxide generation and protein tyrosine phosphorylation [13], indicating that protein tyrosine phosphorylation may be involved in the activation of the NADPH oxidase. In support of this hypothesis, the putative phosphotyrosine phosphatase inhibitor, orthovanadate [14,15], has also been reported to stimulate tyrosine phosphorylation and oxygen consumption in electropermeabilized neutrophils [14].

In cytochalasin B-primed human neutrophils the activation of PLD is required for the generation of superoxide anion [16]. A variety of receptor agonists stimulate PLD activity and superoxide generation in the neutrophil, including fMet-Leu-Phe, platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) ([3,17]; R. Randall, unpublished work). These agents have also been shown to stimulate increases in protein tyrosine phosphorylation in these cells [18–20]. Furthermore, the tyrosine kinase inhibitors erbstatin [21] and ST638 [22] have been reported to inhibit both tyrosine phosphorylation and superoxide generation in fMet-Leu-Phe- and PAF-stimulated neutrophils [19,23]. Such observations have led us to investigate the role of tyrosine phosphorylation in the receptor-mediated activation of PLC and PLD in the human neutrophil.

MATERIALS AND METHODS

[^{3}H]Butan-1-ol, [^{3}H]Ins(1,4,5)P_3, ^{14}C-labelled molecular mass markers (14–200 kDa) and ^{125}I-labelled sheep anti-mouse antibody were purchased from Amersham International, Amersham, Bucks., U.K. The anti-phosphotyrosine antibody 6G9, phosphate-buffered (pH 7.5) saline (PBS) and fetal calf serum were purchased from Gibco. fMet-Leu-Phe, PAF, LTB_4, phorbol 12,13-dibutyrate (PDBu), cytochalasin B, Tween 20, leupeptin, orthovanadate and BSA were from Sigma. Nitrocellulose was obtained from Bio-Rad, Hemel Hempstead, Herts., U.K. All other chemicals and biochemicals were purchased from BDH.

Human neutrophils were purified from whole blood exactly as described [24] and suspended in Hepes-buffered (30 mM, pH 7.2) Hanks balanced salt solution (HBH) at the appropriate cell density. PLD activity was assayed by measuring the incor-

Abbreviations used: PLD, phospholipase D; PKC, protein kinase C; PtdInsP_2-PLC, PtdIns(4,5)P_2-specific phospholipase C; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); LTB_4, leukotriene B_4; PDBu, phorbol 12,13-dibutyrate; HBH, Hepes-buffered (pH 7.2) Hanks balanced salt solution; PBS, phosphate-buffered (pH 7.5) saline (138 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na_2HPO_4 and 1.47 mM-KH_2PO_4, pH 7.5).

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Table 1. Effects of kinase inhibitors on PLD activity

PLD activity was assayed as described in the Materials and methods section. ST271, ST638 and erbstatin were added 30 min before and Ro-31-8220 5 min before stimulation with fMet-Leu-Phe (0.3 μM) or PAF (0.5 μM) for 5 min or PDBu (100 nM) for 30 min. Cells stimulated by fMet-Leu-Phe or PAF were primed with cytochalasin B (5 μM) for 5 min before stimulation. Results are expressed as the concentration of inhibitor required to inhibit the response by 50% (IC_{50}). Values are means ± S.E.M. of three experiments performed in duplicate. n.d., not determined; n.i.e., no inhibitory effect up to 30 μM

Inhibitor	IC_{50} (μM)		
	fMet-Leu-Phe	PAF	PDBu
ST271	6.7±1.7	9.0±1.5	> 100
ST638	25±7.6	25±1.8	> 100
Erbstatin	63±24	n.d.	> 100
Ro-31-8220	n.i.e.	n.d.	0.3

poration of [^{3}H]butan-1-ol into [^{3}H]phosphatidylbutanol as previously described [17], except that [^{3}H]phosphatidylbutanol was located and quantified using an automatic t.l.c. analyser (Tracemaster 40; Berthold). PtdInsP_2-PLC activity was assessed by measuring InsP_3 generation as described [25]. The phosphotyrosine content of neutrophil proteins was assessed by probing Western blots with an anti-phosphotyrosine antibody. Cellular proteins were solubilized in 100 μl of 60 mM-Tris/HCl (pH 6.8) containing SDS (2%, w/v), glycerol (10%, v/v), dithiothreitol (2 mM), Bromophenol Blue (0.001%, w/v), sodium orthovanadate (100 μM) and leupeptin (100 μM) and then boiled for 5 min. The protein samples were separated by SDS/PAGE according to Laemmli [26] on 10% acrylamide gels. The separated proteins were transferred electrophoretically to nitrocellulose membranes, and these blots were washed in PBS for 10 min. The blots were blocked with PBS containing BSA (3%, w/v), Tween 20 (0.2%, v/v) and foetal calf serum (5%, v/v) for 30 min, washed twice at room temperature with PBS containing Tween 20 (0.2%, v/v) and foetal calf serum (5%, v/v), and incubated with mouse monoclonal anti-phosphotyrosine antibody (6G9, 1:500 dilution) for 3 h with constant shaking. After two further washes the blots were incubated for 1 h with ^{125}I-labelled sheep anti-mouse antibody, then thoroughly washed in water, dried and visualized using a PhosphorImager (Molecular Dynamics).

RESULTS

The protein tyrosine kinase inhibitors ST271, ST638 and erbstatin inhibited fMet-Leu-Phe stimulated PLD activation in a concentration-dependent manner (Table 1). The most potent inhibitor (ST271) also inhibited PAF- and LTB_4-stimulated PLD at similar concentrations (Fig. 1). However, PDBu-stimulated PLD was less sensitive to inhibition by ST271, ST638 and erbstatin than was receptor-mediated PLD (Table 1). The PKC inhibitor Ro-31-8220 inhibited PLD activity stimulated by PDBu but not by fMet-Leu-Phe (Table 1).

In contrast to the receptor-mediated activation of PLD, InsP_3 generation stimulated by fMet-Leu-Phe was only weakly inhibited by ST271 at concentrations up to 10 μM (Fig. 2), while at higher concentrations InsP_3 levels began to increase (Fig. 2). This effect of ST271 correlates with the production of InsP_3 in unstimulated cells treated with ST271 (results not shown), and was deemed to be non-specific.

The chemotactic peptide fMet-Leu-Phe increased the phosphotyrosine content of several neutrophil proteins, as determined by Western blotting of SDS/polyacrylamide gels and probing with an anti-phosphotyrosine antibody (Fig. 3). Preliminary experiments (results not shown) revealed that fMet-Leu-Phe-stimulated tyrosine phosphorylation was rapid, and that several proteins

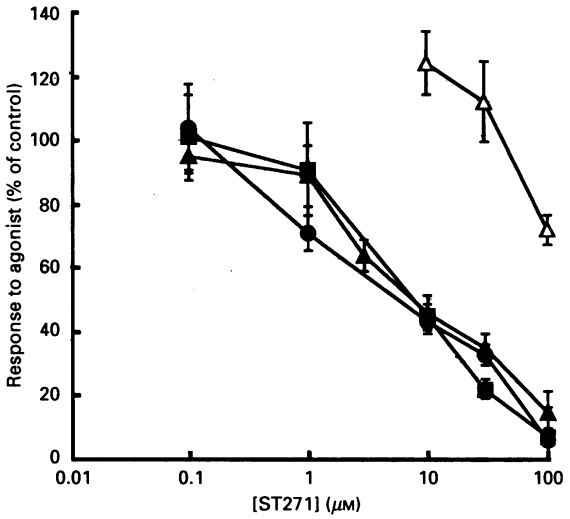

Fig. 1. Inhibition of PLD activity by ST271

PLD activity was assayed as described in the Materials and methods section. Neutrophils were preincubated for 30 min with ST271 before stimulation with fMet-Leu-Phe (0.3 μM, ●), PAF (0.5 μM, ■) or LTB_4 (0.5 μM, ▲) for 5 min, or PDBu (100 nM, △) for 30 min. Cells stimulated by fMet-Leu-Phe, PAF or LTB_4 were primed with cytochalasin B (5 μM) for 5 min before stimulation. Results are means ± S.E.M. of three experiments performed in duplicate. Pretreatment of PDBu-stimulated cells with cytochalasin B did not alter the activity of ST271.

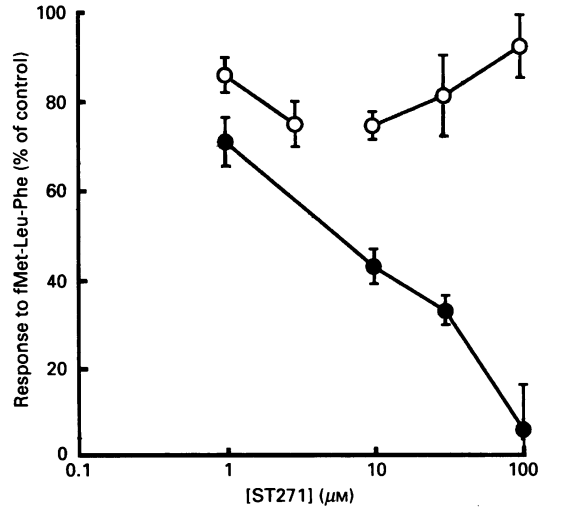

Fig. 2. Effect of ST271 on fMet-Leu-Phe-stimulated PLD and PtdInsP_2-PLC activity

PLD (●) and PtdInsP_2-PLC (○) activities were assayed as described in the Materials and methods section. Neutrophils were preincubated for 30 min with ST271 and for 5 min with cytochalasin B (5 μM) before stimulation with 0.3 μM-fMet-Leu-Phe. Phosphatidylbutanol was measured after a further 5 min and InsP_3 levels were determined after 20 s. Results are means ± S.E.M. of three experiments performed in duplicate (PLD) or five experiments performed in triplicate (PLC).

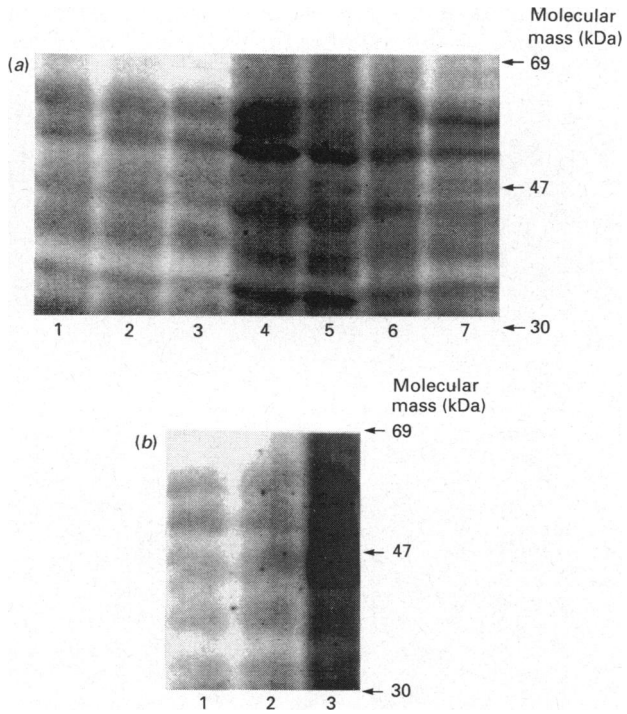

Fig. 3. Effect of ST271 on tyrosine phosphorylation

The phosphotyrosine content of neutrophil proteins was determined by Western blotting as described in the Materials and methods section. Neutrophils were incubated with ST271 where required for 30 min and with cytochalasin B for 5 min before stimulation with fMet-Leu-Phe (0.3 μM) for 1 min or pervanadate (a mixture of 3 mM-H_2O_2 and 300 μM-sodium orthovanadate) for 25 min. The cells were pelleted by centrifugation at 15000 *g* for 2–3 s before the proteins were solubilized. (*a*) Lane 1, no treatment; lane 2, cytochalasin B; lane 3, fMet-Leu-Phe; lane 4, cytochalasin B/fMet-Leu-Phe; lanes 5–7, cytochalasin B/fMet-Leu-Phe + ST271 (1, 10 and 100 μM respectively). (*b*) Lane 1, no treatment; lane 2, cytochalasin B; lane 3, cytochalasin B/pervanadate. Treatment with H_2O_2 alone or orthovanadate alone did not stimulate tyrosine phosphorylation. Molecular masses of standards in kDa are indicated. Results shown are from a single experiment representative of three.

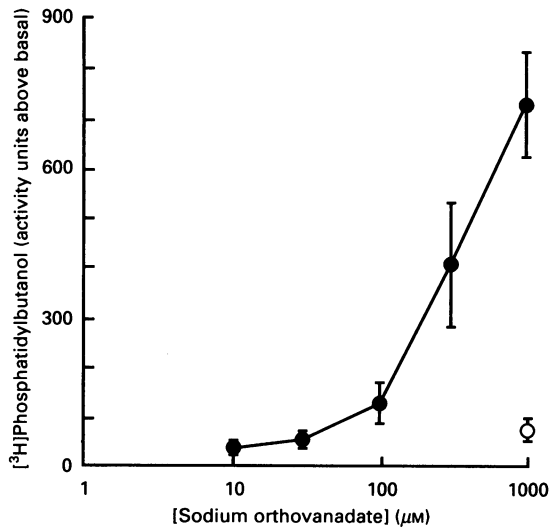

Fig. 4. Effect of pervanadate on PLD activity

PLD activity was assayed as described in the Materials and methods section. Neutrophils were preincubated for 5 min with cytochalasin B (5 μM) before stimulation with increasing concentrations of sodium orthovanadate in the presence (●) or absence (○) of H_2O_2 (3 mM) for 25 min. Results are expressed as activity units, measured by a t.l.c. analyser, above basal levels. The mean basal radioactivity was 315 ± 16 units Average stimulation by fMet-Leu-Phe gave 350 units above basal.

were phosphorylated within 1 min, as reported by other workers [18,19]. Treatment of neutrophils for 30 min with ST271 (1–100 μM) inhibited the protein tyrosine phosphorylation stimulated by fMet-Leu-Phe (Fig. 3).

A mixture of 300 μM-sodium orthovanadate and 3 mM-hydrogen peroxide increased the phosphotyrosine content of many neutrophil proteins (Fig. 3). Under identical conditions this reagent also stimulated PLD activity, in a manner which was dependent on the concentration of orthovanadate (Fig. 4).

DISCUSSION

ST271, ST638 and erbstatin were first identified as inhibitors of the epidermal growth factor receptor tyrosine kinase [21,22]. Previous studies have shown ST271 and ST638 to be relatively specific inhibitors of tyrosine rather than serine/threonine kinases [22,27]. In this study we have shown that they also inhibit PLD stimulated by fMet-Leu-Phe, PAF and LTB_4 in human neutrophils. This inhibition occurred at similar concentrations for all three agonists, demonstrating that these compounds are not acting as receptor antagonists. In contrast, ST271 and ST638 did not inhibit PDBu-stimulated PLD. Unlike stimulation of PLD by receptor agonists, phorbol ester-stimulated PLD activity is not enhanced by pretreatment with cytochalasin B. Inclusion of cytochalasin B did not significantly alter the effect of ST271 on PDBu-stimulated PLD (results not shown). This differential inhibition of receptor-agonist-stimulated and phorbol-ester-stimulated PLD indicates that these compounds do not inhibit PLD *per se*. Their site of action is therefore neither at the receptor nor at the phospholipase, but at a level which is common to the coupling mechanisms of fMet-Leu-Phe, PAF and LTB_4 receptors to PLD. The inhibition of PLD activation by ST271 occurred at concentrations similar to those which inhibited tyrosine phosphorylation. Together, these results suggest that tyrosine kinase activity is intimately involved in the coupling of the fMet-Leu-Phe receptor to PLD. Further indirect evidence for this hypothesis comes from the observation that inhibition of tyrosine phosphatases by pervanadate stimulated both tyrosine phosphorylation and PLD activity.

Pervanadate stimulated a large increase in protein tyrosine phosphorylation, suggesting that phosphotyrosine phosphatase activity is high in the resting neutrophil [14]. Inhibition of phosphatase activity may, therefore, play an important part in stimulating tyrosine phosphorylation, and thus signal transduction, in the neutrophil.

ST271 inhibited the increase in protein tyrosine phosphorylation stimulated by fMet-Leu-Phe, but left InsP_3 production largely unaffected. Furthermore, fMet-Leu-Phe-stimulated InsP_3 production was not dependent on pretreatment with cytochalasin B (results not shown), whereas in the absence of this priming agent, fMet-Leu-Phe did not stimulate tyrosine phosphorylation (Fig. 3). Therefore it would appear that tyrosine phosphorylation is not involved in PtdInsP_2-PLC activation in the neutrophil. This is in contrast to observations in other cell types [10], but is consistent with previous experiments with pertussis toxin which suggest that fMet-Leu-Phe receptors couple to PtdInsP_2-PLC in the neutrophil directly through a GTP-binding protein [12].

It has been suggested that the fMet-Leu-Phe receptor couples to PLD indirectly through the products of PtdInsP_2 hydrolysis

(see above). This would explain the observed sensitivity of fMet-Leu-Phe-stimulated PLD to pertussis toxin [7]. However, the PKC inhibitor Ro-31-8220 did not inhibit fMet-Leu-Phe-stimulated PLD activity, suggesting that, in the neutrophil, activation of PKC by PtdInsP_2-derived diacylglycerol is not involved in the activation of PLD by fMet-Leu-Phe. Similar results have been reported in fMet-Leu-Phe-stimulated HL60 granulocytes with another PKC inhibitor, K252a [6]. These results suggest that in the neutrophil the coupling of receptors to PLD is distinct from their coupling to PtdInsP_2-PLC, and involves tyrosine phosphorylation. The kinases or phosphatases involved may be directly linked to the fMet-Leu-Phe receptor through a pertussis toxin-sensitive GTP-binding protein, or may be regulated by the products of PtdInsP_2 hydrolysis.

The primary amino acid sequence of the fMet-Leu-Phe receptor as reported by Thomas *et al.* [28] shows this receptor to be of a type known to interact with regulatory GTP-binding proteins, and thus it is not a member of the class of receptors with tyrosine kinase activity. Therefore it is likely that non-receptor tyrosine kinases (e.g. hck [29]) are responsible for the activation of PLD by fMet-Leu-Phe in the neutrophil. Evidence from studies in T-lymphocytes suggests that src-like kinases are involved in the coupling of the T-cell receptor to PtdInsP_2-PLCγ type I. The activity of these src-like kinases in the lymphocyte is modulated in turn by the phosphotyrosine phosphatase activity of CD45, a protein which is also present in the neutrophil [11,30,31]. The involvement of CD45 in signal transduction in the neutrophil is suggested by the observation that the level of CD45 at the cell surface is increased by treatment with the priming agent, cytochalasin B, and/or fMet-Leu-Phe [30]. Cytochalasin B enhances many neutrophil responses, but the mechanism of this priming is unknown. Cytochalasin B potentiates PLD activity stimulated by many receptor agonists, but has little effect on PtdInsP_2-PLC activity (results not shown). This observation suggests that cytochalasin B may modulate neutrophil responses through an effect at the level of protein tyrosine phosphorylation.

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